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PATENT APPLICATION

CODON OPTIMIZED SYNTHETIC PLASMIDS

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CODON OPTIMIZED SYNTHETIC PLASMIDS

RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application, Serial Number 60/396,247, entitled "Codon Optimized Synthetic Plasmids," filed on July 16, 2002, the entire content of which is hereby incorporated by reference.

BACKGROUND

[0002] One aspect of the current invention is an optimized nucleic acid delivery vehicle, or synthetic expression plasmid. The synthetic expression plasmid of this invention has reduced components, and has been optimized to increase efficacy, and reduce adverse reactions *in vivo*. In addition to a mammalian gene of interest, a typical nucleic acid delivery vehicle or synthetic expression plasmid contains many structural elements necessary for the *in vitro* amplification of the plasmid in a bacterial host. Consequently, some of the inherent bacterial nucleic acid sequences can cause adverse effects when the amplified plasmid is introduced into a mammalian host. For example, the presence of CpG sequences are known to cause both gene silencing and initiate an immune response in mammals. By utilizing codon optimization, essential bacterial structural elements (e.g. bacterial antibiotic resistant genes) are synthetically constructed and used to replace codons that contained detrimental sequences, but do not effect the final gene product. The current invention involves a "synthetic plasmid backbone" (pAV0201) that provides a clean lineage, which is useful for plasmid supplementation therapy in mammals.

[0003] A plasmid based mammalian expression system is minimally composed of a plasmid backbone, a synthetic delivery promoter in addition to the nucleic acid encoding a therapeutic expression product. A plasmid backbone typically contains a bacterial origin of replication, and a bacterial antibiotic selection gene, which are necessary for the specific growth of only the bacteria that are transformed with the proper plasmid. However, there are plasmids, called mini-circles, that lack both the antibiotic resistance gene and the origin of replication (Darquet et al., 1997; Darquet et al., 1999; Soubrier et al., 1999). The use of *in vitro* amplified expression plasmid DNA (i.e. non-viral expression systems) avoids the risks associated with viral vectors. The non-viral expression systems products generally have low

toxicity due to the use of "species-specific" components for gene delivery, which minimizes the risks of immunogenicity generally associated with viral vectors. One aspect of the current invention is a new, versatile, and codon optimized plasmid based mammalian expression system that will reduce the adverse effects associated with prokaryotic nucleic acid sequences in mammalian hosts. In addition, this new plasmid will constitute the base of a species-specific library of plasmids for expression of hormones or other proteins for agricultural and companion animal applications.

[0004] Codon optimization: Expression of eukaryotic gene products in prokaryotes is sometimes limited by the presence of codons that are infrequently used in *E. coli*. Expression of such genes can be enhanced by systematic substitution of the endogenous codons with codons over represented in highly expressed prokaryotic genes. Although not wanting to be bound by theory, it is commonly thought that rare codons cause pausing of the ribosome. Pausing of the ribosome can lead to a failure to complete the nascent polypeptide chain and a uncoupling of transcription and translation. Additionally, pausing of the ribosome is thought to expose the 3' end of the mRNA to cellular ribonucleases. An invention thought to circumvented such problems for prokaryotic expression of eukaryotic genes was discussed in U.S. Patent No. 6,114,148 issued on September 5, 2000 and titled "High level expression of proteins" with Seed, et al., listed as inventors ("the Seed '148 Patent"). The Seed '148 patent features a synthetic gene that encodes a protein normally expressed in a mammalian cell wherein a non-preferred codon in the natural gene encoding the protein has been replaced by a preferred codon encoding the same amino acid. In contrast, the use of prokaryotic codons in mammalian systems can lead to detrimental effects (e.g. increased immune response). Furthermore, there are species specific differences with codons that are preferred, or less-preferred among species of a genus (Narum et al., 2001). One aspect of the current invention is the codon optimization of modified mammalian gene sequences. Publicly available databases for optimized codons have been referenced in the following articles: Nagata T, Uchijima M, Yoshida A, Kawashima M, Koide Y. Biochem Biophys Res Commun 261:445-51 (1999). Codon optimization effect on translational efficiency of DNA vaccine in mammalian cells: analysis of plasmid DNA encoding a CTL epitope derived from microorganisms; Uchijima, M, Yoshida, A, Nagata, T, Koide, Y. J Immunol 161:5594-9 (1998). Optimization of codon usage of plasmid DNA vaccine is required for the effective MHC class I-restricted T cell responses against an intracellular bacterium; Meetei, AR and

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[0005] As mentioned above, a plasmid backbone typically contains a bacterial origin of replication, and a bacterial antibiotic selection gene, which are necessary for the specific growth of only the bacteria that are transformed with the proper plasmid. However, the nucleotide sequence of the bacterial gene products can adversely affect a mammalian host receiving plasmid DNA. For example, it was desirable to avoid CpG sequences, as these sequences have been shown to cause a recipient host to have an immune response (Manders and Thomas, 2000; Scheule, 2000) to plasmids as well as possible gene silencing (Shi et al., 2002; Shiraishi et al., 2002). Thus, the DNA coding regions of any expressed genes avoid the "cg" sequence, without changing the amino acid sequence. Another aspect of the current invention involves the removal of unnecessary DNA sequences that were left over from prior cloning procedures. As a result of codon optimization, and removal of unnecessary DNA sequences, a synthetically generated plasmid backbone ("pAV0201") with a unique cloning site that was constructed to generate a clean lineage of plasmid, which will be useful for plasmid mediated gene supplementation.

[0006] **Growth Hormone ("GH") and Immune Function:** Another aspect of the current invention is utilizing the synthetically generated plasmid backbone pAV0201 for plasmid mediated gene supplementation. The central role of growth hormone ("GH") is controlling somatic growth in humans and other vertebrates, and the physiologically relevant pathways regulating GH secretion from the pituitary is well known (Berneis and Keller, 1996). The GH production pathway is composed of a series of interdependent genes whose products are required for normal growth (Cuttler, 1996). The GH pathway genes include: (1) ligands, such as GH and insulin-like growth factor-I ("IGF-I"); (2) transcription factors such as prophet of pit 1, or prop 1, and pit 1: (3) agonists and antagonists, such as growth hormone releasing hormone ("GHRH") and somatostatin ("SS"), respectively; and (4) receptors, such as GHRH receptor ("GHRH-R") and the GH receptor ("GH-R"). These genes are expressed in different organs and tissues, including the hypothalamus, pituitary, liver, and bone. Effective and regulated expression of the GH pathway is essential for optimal linear growth, as well as homeostasis of carbohydrate, protein, and fat metabolism GH synthesis and secretion from the anterior pituitary is stimulated by GHRH and inhibited by somatostatin, both hypothalamic hormones (Frohman et al., 1992). GH increases production of IGF-I, primarily in the liver, and other target organs. IGF-I and GH, in turn, feedback on the hypothalamus and pituitary to inhibit GHRH and GH release. GH elicits both direct and indirect actions on peripheral tissues, the indirect effects being mediated mainly by IGF-I.

[0007] The immune function is modulated by IGF-I (Geffner, 1997; LeRoith et al., 1996), which has two major effects on B cell development: potentiation and maturation, and as a B-cell proliferation cofactor that works together with interlukin-7 ("IL-7"). These activities were identified through the use of anti-IGF-I antibodies, antisense sequences to IGF-I, and the use of recombinant IGF-I to substitute for the activity. There is evidence that macrophages are a rich source of IGF-I. The treatment of mice with recombinant IGF-I confirmed these observations as it increased the number of pre-B and mature B cells in bone marrow. The mature B cell remained sensitive to IGF-I as immunoglobulin production was also stimulated by IGF-I in vitro and in vivo.

[0008] The production of recombinant proteins in the last 2 decades provided a useful tool for the treatment of many diverse conditions. For example, GH-deficiencies in short stature children, anabolic agent in burn, sepsis, and AIDS patients (Carrel and Allen,

2000; Hart et al., 2001; Lal et al., 2000; Mulligan et al., 1999). However, resistance to GH action has been reported in malnutrition and infection (Kotzmann et al., 2001). Long-term studies on transgenic animals and in patients undergoing GH therapies have shown no correlation in between GH or IGF-I therapy and cancer development. GH replacement therapy is widely used clinically, with beneficial effects, but therapy is associated with several disadvantages (Blethen, 1995): GH must be administered subcutaneously or intramuscularly once a day to three times a week for months, or usually years; insulin resistance and impaired glucose tolerance (Burgert et al., 2002); accelerated bone epiphysis growth and closure in pediatric patients (Blethen and Rundle, 1996).

[0009] In contrast, essentially no side effects have been reported for recombinant GHRH therapies. Extracranially secreted GHRH, as mature peptide or truncated molecules (as seen with pancreatic islet cell tumors and variously located carcinoids) are often biologically active and can even produce acromegaly (Faglia et al., 1992; Melmed, 1991). Administration of recombinant GHRH to GH-deficient children or adult humans augments IGF-I levels, increases GH secretion proportionally to the GHRH dose, yet still invokes a response to bolus doses of recombinant GHRH (Bercu et al., 1997). Thus, GHRH administration represents a more physiological alternative of increasing subnormal GH and IGF-I levels (Corpas et al., 1993b).

[0010] GH is released in a distinctive pulsatile pattern that has profound importance for its biological activity. Secretion of GH is stimulated by the GHRH, and inhibited by somatostatin, and both hypothalamic hormones. GH pulses are a result of GHRH secretion that is associated with a diminution or withdrawal of somatostatin secretion. In addition, the pulse generator mechanism is timed by GH-negative feedback. The endogenous rhythm of GH secretion becomes entrained to the imposed rhythm of exogenous GH administration. Effective and regulated expression of the GH and insulin-like growth factor-I ("IGF-I") pathway is essential for optimal linear growth, homeostasis of carbohydrate, protein, and fat metabolism, and for providing a positive nitrogen balance. Numerous studies in humans, sheep or pigs showed that continuous infusion with recombinant GHRH protein restores the normal GH pattern without desensitizing GHRH receptors or depleting GH supplies as this system is capable of feed-back regulation, which is abolished in the GH therapies. Although recombinant GHRH protein therapy entrains and stimulates normal

cyclical GH secretion with virtually no side effects (Duck et al., 1992), the short half-life of GHRH in vivo requires frequent (one to three times a day) intravenous, subcutaneous or intranasal (requiring 300-fold higher dose) administration (Evans et al., 1985). Thus, as a chronic treatment, GHRH administration is not practical.

[0011] Wild type GHRH has a relatively short half-life in the circulatory system, both in humans and in farm animals (Frohman et al., 1986). After 60 minutes of incubation in plasma 95% of the GHRH(1-44)NH_2 is degraded, while incubation of the shorter (1-40)OH form of the hormone, under similar conditions, shows only a 77% degradation of the peptide after 60 minutes of incubation (Frohman et al., 1989a). Incorporation of cDNA coding for a particular protease-resistant GHRH analog in a gene therapy vector results in a molecule with a longer half-life in serum (Draghia-Akli et al., 1999), increased potency, and provides greater GH release in plasmid-injected animals as described in in U.S. Patent 6,551,996 that was issued on April 23, 2003 titled "Super Active Porcine Growth Hormone Releasing Hormone Analog" with Schwartz, et al., listed as inventors, ("the Schwartz '996 Patent"), the entire content is herein incorporated by reference. The Schwartz '996 Patent teaches that an application of a GHRH analog containing mutations that improve the ability to elicit the release of growth hormone. In addition, the Schwartz '996 Patent relates to the treatment of growth deficiencies; the improvement of growth performance; the stimulation of production of growth hormone in an animal at a greater level than that associated with normal growth; and the enhancement of growth utilizing the administration of growth hormone releasing hormone analog and is herein incorporated by reference. Mutagenesis via amino acid replacement of protease sensitive amino acids prolongs the serum half-life of the GHRH molecule. Furthermore, the enhancement of biological activity of GHRH is achieved by using super-active analogs that may increase its binding affinity to specific receptors as described in the Schwartz '996 Patent.

[0012] Extracranially secreted GHRH, as processed protein species GHRH(l-40) hydroxy or GHRH(l-44) amide or even as shorter truncated molecules, are biological active. It has been reported that a low level of GHRH (100 pg/ml) in the blood supply stimulates GH secretion (Corpas et al., 1993a). Direct plasmid DNA gene transfer is currently the basis of many emerging therapeutic strategies and thus does not require viral genes or lipid particles (Aihara and Miyazaki, 1998; Lesbordes et al., 2002). Skeletal muscle is a target tissue

because muscle fiber has a long life span and can be transduced by circular DNA plasmids that express over months or years in an immunocompetent host (Danko and Wolff, 1994; Wolff et al., 1992). Previous reports demonstrated that human GHRH cDNA could be delivered to muscle by an injectable myogenic expression vector in mice where it transiently stimulated GH secretion over a period of two weeks in immunocompetent mice (Draghia-Akli et al., 1997), and for 5 month in immunodeficient mice (Draghia-Akli et al., 2002)(human hormones are immunogenic in normal immunocompetent rodents, and transgene expression is transitory in these cases).

[0013] U.S. Patent No. 5,061,690 issued on October 29, 1991 and titled "Method for increasing milk production in mammals and/or increasing the birth weight of their newborn and improving postnatal growth " with Kann, et al., listed as inventors, ("the Kann '690 patent"). The Kann '690 patent is directed toward increasing both birth weight and milk production by supplying to pregnant female mammals an effective amount of human GHRH or one of it analogs for 10-20 days. Application of the analogs lasts only throughout the lactation period. However, multiple administrations are presented, and there is no teachings regarding administration of the growth hormone releasing hormone a nucleic acid delivery vehicle or a codon optimized synthetic mammalian expression plasmid.

[0014] U.S. Patent No. 5,134,120 issued on July 28, 1992 and titled "Use of growth hormone to enhance porcine weight gain" with Boyd, et al., listed as inventors, (" the Boyd '120 patent"); and U.S. Patent No. 5,292,721 issued on March 8, 1994 and titled "Use of growth hormone to enhance porcine fetal energy and sow lactation performance" with Boyd, et al., listed as inventors, ("the Boyd '721 patent"). Both the Boyd ' 120, and Boyd 721 patent teach that by deliberately increasing growth hormone in swine during the last 2 weeks of pregnancy through a 3 week lactation resulted in the newborn piglets having marked enhancement of the ability to maintain plasma concentrations of glucose and free fatty acids when fasted after birth. In addition, the Boyd '120 and Boyd '721 patents teach that treatment of the sow during lactation results in increased milk fat in the colostrum and an increased milk yield. These effects are important in enhancing survivability of newborn pigs and weight gain prior to weaning. However Boyd '120 and Boyd '721 patents provide no teachings regarding administration of the growth hormone releasing hormone a nucleic acid delivery vehicle or a codon optimized synthetic mammalian expression plasmid.

[0015] In summary, previous studies have shown that it is possible to treat various disease conditions in a limited capacity utilizing recombinant protein technology, but these treatments have some significant drawbacks. It has also been taught that nucleic acid expression plasmids that encode recombinant proteins are viable solutions to the problems of frequent injections and high cost of traditional recombinant therapy. However, the nucleic acid expression plasmids also have some drawbacks when injected into a mammalian host. The synthetic plasmids of this invention have reduced components, and have been codon optimized to increase efficacy, and reduce adverse reactions *in vivo*. The introduction of point mutations in to the encoded recombinant proteins was a significant step forward in producing proteins that are more stable *in vivo* than the wild type counterparts. Since there is a need in the art to expanded treatments for subjects with a disease by utilizing nucleic acid expression constructs that are delivered into a subject and express stable therapeutic proteins *in vivo*, the combination of codon optimization of an encoded therapeutic mammalian gene in an optimized plasmid backbone will further enhance the art of plasmid mediated gene supplementation.

SUMMARY

[0016] One aspect of the current invention is an optimized synthetic mammalian expression plasmid (e.g. pAV0201). This new plasmid comprises a therapeutic element, and a replication element. The therapeutic element of the new plasmid comprises a eukaryotic promoter; a 5' untranslated region ("UTR"); a codon-optimized-eukaryotic therapeutic gene sequence; and a polyadenylation signal. The therapeutic elements of this plasmid are operatively linked and located in a first operatively-linked arrangement. Additionally, the optimized synthetic mammalian expression plasmid comprises replication elements, wherein the replication elements are operatively linked and located in a second operatively-linked arrangement. The replication elements comprise a selectable marker gene promoter, a ribosomal binding site, a optimized selectable marker gene sequence, and an origin of replication. The first-operatively-linked arrangement and the second-operatively-linked arrangement comprise a circular structure of the codon optimized synthetic mammalian expression plasmid.

[0017] In preferred embodiments, the synthetic mammalian expression plasmid comprises a pUC-18 prokaryotic origin of replication sequence. However, the origin of replication may also comprise an autonomously replication sequence ("ARS"). In a preferred embodiment, the optimized prokaryotic antibiotic resistant gene comprises kanamycin. In another preferred embodiment, the poly adenylation signal ("PolyA") comprises a human growth hormone ("hGH") poly A signal, and a hGH 5' untranslated region ("5'UTR"). The codon optimized mammalian therapeutic gene sequence comprises a sequence that encodes a modified species specific growth hormone releasing hormone ("GHRH"). In preferred embodiments, the codon optimized sequence comprises porcine, mouse, rat, bovine, ovine, and chicken GHRH (e.g. SeqID#4, SeqID#5; SeqID#6; SeqID#7; SeqID#8; and SeqID#9). Similarly, species specific, and codon optimized plasmids are disclosed (e.g. SEQID#17; SEQID#18; SEQID#19; SEQID#20; and SEQID#21).

[0018] Another aspect of the current invention is a method for plasmid mediated gene supplementation that comprises delivering a codon optimized synthetic mammalian expression plasmid into a subject. The codon optimized synthetic mammalian expression plasmid encodes a growth hormone releasing hormone ("GHRH") or functional biological

equivalent in the subject. The method of delivering the codon optimized synthetic mammalian expression plasmid into the cells of the subject is via electroporation. In a preferred embodiment, the cells of the subject can be somatic cells, stem cells, or germ cells. The codon optimized synthetic mammalian expression plasmids consisting of SeqID#17, SeqID#18, SeqID#19, SeqID#20, and SeqID#21 have been contemplated by the inventors. The encoded GHRH is a biologically active polypeptide; and the encoded functional biological equivalent of GHRH is a polypeptide that has been engineered to contain a distinct amino acid sequence while simultaneously having similar or improved biologically activity when compared to the GHRH polypeptide. One result of expressing the encoded GHRH or functional biological equivalent thereof in a subject is the facilitation of growth hormone ("GH") secretion in the subject.

BRIEF DESCRIPTION OF FIGURES

[0019] Figure 1 shows a general map of a plasmid construct (pAV0125, this plasmid contains the porcine modified HV-GHRH sequence) used prior construction of an optimized synthetic plasmid of the current invention;

[0020] Figure 2 shows a general map of a synthetic plasmid construct (pAV0201, this construct contains the porcine modified GHRH called HV-GHRH) of the current invention, which contains codon optimization;

[0021] Figure 3 shows the optimized nucleic acid sequence for the kanamycin gene and the corresponding translated amino acid sequence;

[0022] Figure 4 shows a schematic map of a 228 bp synthetic nucleic acid sequence for mouse GHRH ("mGHRH");

[0023] Figure 5 shows the optimized nucleic acid sequence for the mGHRH gene and the corresponding translated amino acid sequence;

[0024] Figure 6 shows the optimized nucleic acid sequence for the original mGHRH gene ("GHRH-m-ori"), and the optimized mGHRH gene ("GHRH-m-opt") after removing some CpG islands and other motifs that can decrease protein expression, the changes did not effect the amino acid sequence;

[0025] Figure 7 shows a comparison of the translated amino acid sequence from the original ("GHRH-m-Ori") and optimized nucleic acid sequence for the mouse GHRH gene ("GHRH-m-Opti");

[0026] Figure 8 shows a schematic map of a 231 bp synthetic nucleic acid sequence for rat GHRH ("rGHRH");

[0027] Figure 9 shows the optimized nucleic acid sequence for the rGHRH gene and the corresponding translated amino acid sequence;

[0028] Figure 10 shows the optimized nucleic acid sequence for the original rGHRH gene ("GHRH-R-ori"), and the optimized rGHRH gene ("GHRH-R-opt") after removing some CpG islands and other motifs that can decrease protein expression, the changes did not effect the amino acid sequence;

[0029] Figure 11 shows a comparison of the translated amino acid sequence from the original ("GHRH-R-Ori") and optimized nucleic acid sequence for the rat GHRH gene ("GHRH-R-Opti");

[0030] Figure 12 shows a schematic map of a 222 bp synthetic nucleic acid sequence for bovine GHRH ("bGHRH");

[0031] Figure 13 shows the optimized nucleic acid sequence for the bGHRH gene and the corresponding translated amino acid sequence;

[0032] Figure 14 shows the optimized nucleic acid sequence for the original bGHRH gene ("GHRH-B-ori"), and the optimized bGHRH gene ("GHRH-B-opt") after removing some CpG islands and other motifs that can decrease protein expression, the changes did not effect the amino acid sequence;

[0033] Figure 15 shows a comparison of the translated amino acid sequence from the original ("GHRH-B-Ori") and optimized nucleic acid sequence for the bovine GHRH gene ("GHRH-B-Opti");

[0034] Figure 16 shows a schematic map of a 222 bp synthetic nucleic acid sequence for ovine GHRH ("oGHRH");

[0035] Figure 17 shows the optimized nucleic acid sequence for the oGHRH gene and the corresponding translated amino acid sequence;

[0036] Figure 18 shows the optimized nucleic acid sequence for the original oGHRH gene ("GHRH-O-ori"), and the optimized oGHRH gene ("GHRH-O-opt") after removing some CpG islands and other motifs that can decrease protein expression, the changes did not effect the amino acid sequence;

[0037] Figure 19 shows a comparison of the translated amino acid sequence from the original ("GHRH-O-Ori") and optimized nucleic acid sequence for the ovine GHRH gene ("GHRH-O-Opti");

[0038] Figure 20 shows a schematic map of a 234 bp synthetic nucleic acid sequence for chicken GHRH ("cGHRH");

[0039] Figure 21 shows the optimized nucleic acid sequence for the cGHRH gene and the corresponding translated amino acid sequence;

[0040] Figure 22 shows the optimized nucleic acid sequence for the original cGHRH gene ("GHRH-Chi-ori"), and the optimized cGHRH gene ("GHRH-Chi-opt") after removing some CpG islands and other motifs that can decrease protein expression, the changes did not effect the amino acid sequence;

[0041] Figure 23 shows a comparison of the translated amino acid sequence from the original ("GHRH-Chi-Ori") and optimized nucleic acid sequence for the chicken GHRH gene ("GHRH-Chi-Opti");

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

[0042] *Terms:*

[0043] The term "coding region" as used herein refers to any portion of the DNA sequence that is transcribed into messenger RNA (mRNA) and then translated into a sequence of amino acids characteristic of a specific polypeptide.

[0044] The term "analog" as used herein includes any mutant of GHRH, or synthetic or naturally occurring peptide fragments of GHRH.

[0045] The term "codon" as used herein refers to any group of three consecutive nucleotide bases in a given messenger RNA molecule, or coding strand of DNA that specifies a particular amino-acid, a starting or stopping signal for translation. The term codon also refers to base triplets in a DNA strand.

[0046] The term "delivery" as used herein is defined as a means of introducing a material into a subject, a cell or any recipient, by means of chemical or biological process, injection, mixing, electroporation, sonoporation, or combination thereof, either under or without pressure.

[0047] The term "encoded GHRH" as used herein is a biologically active polypeptide.

[0048] The term "functional biological equivalent" of GHRH as used herein is a polypeptide that has been engineered to contain a distinct amino acid sequence while simultaneously having similar or improved biologically activity when compared to the GHRH polypeptide.

[0049] The term "heterologous nucleic acid sequence" as used herein is defined as a DNA sequence consisting of differing regulatory and expression elements.

[0050] The term "growth hormone" ("GH") as used herein is defined as a hormone that relates to growth and acts as a chemical messenger to exert its action on a target cell.

[0051] The term "growth hormone releasing hormone" ("GHRH") as used herein is defined as a hormone that facilitates or stimulates release of growth hormone, and in a lesser extent other pituitary hormones, as prolactin.

[0052] The term "non-optimized codon" as used herein refers to a codon that does not have a match codon frequencies in target and host organisms. The non-optimized codons of this invention were determined using Aptagen's Gene Forge® codon optimization and custom gene synthesis platform (Aptagen, Inc., 2190 Fox Mill Rd. Suite 300, Herndon, VA 20171). Other publicly available databases for optimized codons are available and will work equally as well.

[0053] The term "nucleic acid expression construct" as used herein refers to any type of genetic construct comprising a nucleic acid coding for a RNA capable of being transcribed. The term "expression vector" or "expression plasmid" can also be used interchangeably.

[0054] The term "operatively linked" as used herein refers to elements or structures in a nucleic acid sequence that are linked by operative ability and not physical location. The elements or structures are capable of, or characterized by accomplishing a desired operation. It is recognized by one of ordinary skill in the art that it is not necessary for elements or structures in a nucleic acid sequence to be in a tandem or adjacent order to be operatively linked.

[0055] The term "optimized codon" as used herein refers to a codon that has a match codon frequencies in target and host organisms, but does not alter the amino acid sequence of the original translated protein. The optimized codons of this invention were determined using Aptagen's Gene Forge® codon optimization and custom gene synthesis platform (Aptagen, Inc., 2190 Fox Mill Rd. Suite 300, Herndon, VA 20171). Other publicly available databases for optimized codons are available and will work equally as well.

[0056] The term "optimized nucleic acid delivery vehicle" as used herein refers to any vector that delivers a nucleic acid into a cell or organism wherein at least one of the codons has been optimized for expression in a host organism. The term "synthetic expression

plasmid" can also be used interchangeably with the term optimized nucleic acid delivery vehicle.

[0057] The term "promoter" as used herein refers to a sequence of DNA that directs the transcription of a gene. A promoter may direct the transcription of a prokaryotic or eukaryotic gene. A promoter may be "inducible", initiating transcription in response to an inducing agent or, in contrast, a promoter may be "constitutive", whereby an inducing agent does not regulate the rate of transcription. A promoter may be regulated in a tissue-specific or tissue-preferred manner, such that it is only active in transcribing the operable linked coding region in a specific tissue type or types.

[0058] The term "replication element" as used herein comprises nucleic acid sequences that will lead to replication of a plasmid in a specified host. One skilled in the art of molecular biology will recognize that the replication element may include, but is not limited to a selectable marker gene promoter, a ribosomal binding site, a selectable marker gene sequence, and a origin of replication.

[0059] The term "subject" as used herein refers to any species of the animal kingdom. In preferred embodiments it refers more specifically to humans and animals used for: pets (e.g. cats, dogs, etc.); work (e.g. horses, cows, etc.); food (chicken, fish, lambs, pigs, etc); and all others known in the art.

[0060] The term "therapeutic element" as used herein comprises nucleic acid sequences that will lead to an *in vivo* expression of an encoded gene product. One skilled in the art of molecular biology will recognize that the therapeutic element may include, but is not limited to a promoter sequence, a poly A sequence, or a 3' or 5' UTR.

[0061] The term "vector" as used herein refers to any vehicle that delivers a nucleic acid into a cell or organism. Examples include plasmid vectors, viral vectors, liposomes, or cationic lipids.

[0062] The new synthetic constructs of the current invention are injected intramuscularly into a correspondent species. For example, the bovine GHRH ("bGHRH") construct is utilized in cows, and ovine GHRH ("oGHRH") construct is utilized in sheep.

Although not wanting to be bound by theory, the ovine GHRH will be produced by the sheep muscle fibers, and then delivered into the circulatory system. The circulating hormone will enhance the synthesis and secretion of ovine growth hormone in the anterior pituitary. The new synthetic constructs can promote long-term expression because the new plasmid backbone lacks CpG islands and other bacterial components that alert the immune system of the presence of a foreign antigen. By decreasing the immune response against the plasmid fragment and its products can function in the muscle cells for longer durations of time, which lowers cost of treatment by decreasing the number of treatments. Furthermore, the usage of species-specific transgene will ensure long term expression by the lack of neutralizing antibodies against a foreign GHRH.

[0063] **Plasmid mediated gene supplementation.** The delivery of specific genes to somatic tissue in a manner that can correct inborn or acquired deficiencies and imbalances has been demonstrated in prior art. Plasmid mediated gene supplementation offers a number of advantages over the administration of recombinant proteins. These advantages include the conservation of native protein structure, improved biological activity, avoidance of systemic toxicities, and avoidance of infectious and toxic impurities. In addition, plasmid mediated gene supplementation allows for prolonged exposure to the protein in the therapeutic range, because the newly secreted protein is present continuously in the blood circulation.

[0064] Although not wanting to be bound by theory, the primary limitation of using a recombinant protein is the limited availability of protein after each administration. Plasmid mediated gene supplementation using injectable DNA plasmid expression vectors overcomes this drawback, because a single injection into the subject's skeletal muscle permits physiologic expression for extensive periods of time. Injection of the vectors can promote the production of enzymes and hormones in animals in a manner that more closely mimics the natural process. Furthermore, among the non-viral techniques for gene transfer *in vivo*, the direct injection of plasmid DNA into muscle tissue is simple, inexpensive, and safe.

[0065] In a plasmid based expression system, a non-viral gene vector may be composed of a synthetic gene delivery system in addition to the nucleic acid encoding a therapeutic gene product. In this way, the risks associated with the use of most viral vectors can be avoided. Additionally, no integration of plasmid sequences into host chromosomes has

been reported *in vivo* to date, so that this type of gene transfer should neither activate oncogenes nor inactivate tumor suppressor genes. As episomal systems residing outside the chromosomes, plasmids have defined pharmacokinetics and elimination profiles, leading to a finite duration of gene expression in target tissues.

[0066] One aspect of the current invention is a new, versatile, and codon optimized plasmid based mammalian expression system that will reduce the adverse effects associated with prokaryotic nucleic acid sequences in mammalian hosts. In addition, this new plasmid will constitute the base of a species-specific library of plasmids for expression of hormones or other proteins for agricultural and companion animal applications. The synthetic expression plasmid of this invention has reduced components, and has been optimized to increase efficacy, and reduce adverse reactions *in vivo*. In addition to a mammalian gene of interest, a typical nucleic acid delivery vehicle or synthetic expression plasmid contains many structural elements useful for the *in vitro* amplification of the plasmid in a bacterial host. Consequently, some of the inherent bacterial nucleic acid sequences can cause adverse effects when the amplified plasmid is introduced into a mammalian host. For example, the presence of CpG sequences are known to cause both gene silencing and initiate an immune response in mammals. By utilizing codon optimization, essential bacterial structural elements (e.g. bacterial antibiotic resistant genes) are synthetically constructed and used to replace codons that contained detrimental sequences, but do not effect the final gene product. The current invention involves a "synthetic plasmid backbone" (pAV0201) that provides a clean lineage, which is useful for plasmid supplementation therapy in mammals.

[0067] A plasmid based mammalian expression system is minimally composed of a plasmid backbone, a synthetic delivery promoter in addition to the nucleic acid encoding a therapeutic expression product. A plasmid backbone typically contains a bacterial origin of replication, and a bacterial antibiotic selection gene, which are necessary for the specific growth of only the bacteria that are transformed with the proper plasmid. However, there are plasmids that lack both the antibiotic resistance gene and the origin of replication, such plasmids are called mini-circles (Darquet et al., 1997; Darquet et al., 1999; Soubrier et al., 1999). The use of *in vitro* amplified expression plasmid DNA (i.e. non-viral expression systems) avoids the risks associated with viral vectors. The non-viral expression systems products generally have low toxicity due to the use of "species-specific" components for gene

delivery, which minimizes the risks of immunogenicity generally associated with viral vectors. One aspect of the current invention is a new, versatile, and codon optimized plasmid based mammalian expression system, which will constitute the base of a species-specific library of plasmids for expression of hormones or other proteins for agricultural and companion animal applications. For example, optimized synthetic sequences can be produced such that codon frequencies are matched in target and host organisms to ensure proper folding. A bias of GC content can be used to increase mRNA stability or reduce secondary structures. Tandem repeat codons or base runs that may impair the gene can be minimized with codon optimization. Modification of ribosome binding sites and mRNA degradation sites can be utilized. Optimization can also reduce or eliminate problem secondary structures within the transcribed mRNA.

[0068] **Vectors.** One skilled in the art recognizes that expression vectors derived from various bacterial plasmids may be used for delivery of nucleotide sequences to a targeted organ, tissue or cell population. Methods which are well known to those skilled in the art can be used to construct recombinant vectors that will express a gene of interest or a gene encoding a growth hormone releasing hormone analog. Transient expression may last for a month or more with a non-replicating vector and even longer if appropriate replication elements are a part of the vector system, wherein the term "vector" is used to refer to a carrier nucleic acid molecule into which a nucleic acid sequence can be inserted for introduction into a cell where the vector can be replicated and the nucleic acid sequence can be expressed. The term vector can also be referred to as a nucleic acid construct. A nucleic acid sequence can be "exogenous," which means that it is foreign to the cell into which the vector is being introduced or that the sequence is homologous to a sequence in the cell but in a position within the host cell nucleic acid in which the sequence is ordinarily not found. Vectors include plasmids, cosmids, and artificial chromosomes (*e.g.*, YACs). One of skill in the art would be well equipped to construct a vector through standard recombinant techniques, which are described in Maniatis et al., 1988 the entirety is incorporated herein by reference. The selected expressed nucleic acid sequences of a constructed vector could then be codon optimized as described below.

[0069] The term "expression vector" refers to a vector or nucleic acid expression construct containing a nucleic acid sequence coding for at least part of a gene product capable

of being transcribed. In a specific embodiment the nucleic acid sequence encodes part or all of GHRH. In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. In other cases, these sequences are not translated, for example, in the production of antisense molecules or ribozymes. Expression vectors can contain a variety of "control sequences," which refer to nucleic acid sequences necessary for the transcription and possibly translation of an operably linked coding sequence in a particular host organism. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well and are described *infra*.

[0070] In a preferred embodiment, the nucleic acid construction construct or vector of the present invention is a plasmid which comprises a synthetic myogenic (muscle-specific) promoter, a synthetic nucleotide sequence encoding a growth hormone releasing hormone or its analog, and a 3′ untranslated region. In other alternative embodiments, optimized porcine growth hormone, optimized human growth hormone, optimized mouse growth hormone, optimized rat growth hormone, optimized bovine growth hormone, optimized ovine growth hormone, optimized chicken growth hormone, or skeletal alpha actin 3′ untranslated regions are utilized in the vector.

[0071] **Promoters and Enhancers.** A "promoter" is a control sequence that is a region of a nucleic acid sequence at which initiation and rate of transcription are controlled. It may contain genetic elements at which regulatory proteins and molecules may bind such as RNA polymerase and other transcription factors. The phrases "operatively positioned," "operatively linked," "under control," and "under transcriptional control" mean that a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence to control transcriptional initiation and/or expression of that sequence. A promoter may or may not be used in conjunction with an "enhancer," which refers to a cis-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence.

[0072] A promoter may be one of naturally-coding sequences located upstream of the coding segment and/or exon. Such a promoter can be referred to as "endogenous." Similarly, an enhancer may be one naturally associated with a nucleic acid sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding nucleic acid segment under the control of a recombinant or

heterologous promoter, which refers to a promoter that is not normally associated with a nucleic acid sequence in its natural environment. A recombinant or heterologous enhancer refers also to an enhancer not normally associated with a nucleic acid sequence in its natural environment. Such promoters or enhancers may include promoters or enhancers of other genes, and promoters or enhancers isolated from any other prokaryotic, viral, or eukaryotic cell, and promoters or enhancers not "naturally occurring," *i.e.*, containing different elements of different transcriptional regulatory regions, and/or mutations that alter expression. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including PCR™. Furthermore, it is contemplated the control sequences that direct transcription and/or expression of sequences within non-nuclear organelles such as mitochondria, chloroplasts, and the like, can be employed as well.

[0073] Naturally, it will be important to employ a promoter and/or enhancer that effectively directs the expression of the DNA segment in the cell type, organelle, and organism chosen for expression. Those of skill in the art of molecular biology generally know the use of promoters, enhancers, and cell type combinations for protein expression. The promoters employed may be constitutive, tissue-specific, inducible, and/or useful under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins and/or peptides. The promoter may be heterologous or endogenous. In a specific embodiment the promoter is a synthetic myogenic promoter.

[0074] The identity of tissue-specific promoters or elements, as well as assays to characterize their activity, is well known to those of skill in the art. Examples of such regions include the human LIMK2 gene, the somatostatin receptor 2 gene, murine epididymal retinoic acid-binding gene, human CD4, mouse alpha2 (XI) collagen, D1A dopamine receptor gene, insulin-like growth factor II, human platelet endothelial cell adhesion molecule-1.

[0075] **Initiation Signals and Internal Ribosome Binding Sites.** A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon or adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of

ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be "in-frame" with the reading frame of the desired coding sequence to ensure translation of the entire insert. The exogenous translational control signals and initiation codons can be either natural or synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements.

[0076] In certain embodiments of the invention, the use of internal ribosome entry sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5' methylated Cap dependent translation and begin translation at internal sites. IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described, as well an IRES from a mammalian message. IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message.

[0077] **Multiple Cloning Sites.** Vectors can include a multiple cloning site ("MCS"), which is a nucleic acid region that contains multiple restriction enzyme sites, any of which can be used in conjunction with standard recombinant technology to digest the vector. "Restriction enzyme digestion" refers to catalytic cleavage of a nucleic acid molecule with an enzyme that functions only at specific locations in a nucleic acid molecule. Many of these restriction enzymes are commercially available. Use of such enzymes is widely understood by those of skill in the art. Frequently, a vector is linearized or fragmented using a restriction enzyme that cuts within the MCS to enable exogenous sequences to be ligated to the vector. "Ligation" refers to the process of forming phosphodiester bonds between two nucleic acid fragments, which may or may not be contiguous with each other. Techniques involving restriction enzymes and ligation reactions are well known to those of skill in the art of recombinant technology.

[0078] **Splicing Sites.** Most transcribed eukaryotic RNA molecules will undergo RNA splicing to remove introns from the primary transcripts. Vectors containing

genomic eukaryotic sequences may require donor and/or acceptor splicing sites to ensure proper processing of the transcript for protein expression.

[0079] **Polyadenylation Signals.** In expression, one will typically include a polyadenylation signal to effect proper polyadenylation of the transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and/or any such sequence may be employed. Preferred embodiments include the bovine or human growth hormone polyadenylation signal, convenient and/or known to function well in various target cells. Also contemplated as an element of the expression cassette is a transcriptional termination site. These elements can serve to enhance message levels and/or to minimize read through from the cassette into other sequences.

[0080] **Origins of Replication.** In order to propagate a vector in a host cell, it may contain one or more origins of replication sites (often termed "ori"), which is a specific nucleic acid sequence at which replication is initiated. Alternatively an autonomously replicating sequence (ARS) can be employed if the host cell is yeast.

[0081] **Selectable and Screenable Markers.** In certain embodiments of the invention, the cells that contain the nucleic acid construct of the present invention may be identified *in vitro* or *in vivo* by including a marker in the expression vector. Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the expression vector. Generally, a selectable marker is one that confers a property that allows for selection. A positive selectable marker is one in which the presence of the marker allows for its selection, while a negative selectable marker is one in which its presence prevents its selection. An example of a positive selectable marker is a drug resistance marker, such as the antibiotic resistance gene on the plasmid constructs (such as kanamycin, ampicylin, gentamycin, tetracycline, or chloramphenicol).

[0082] Usually the inclusion of a drug selection marker aids in the cloning and identification of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selectable markers. In addition to markers conferring a phenotype that allows for the discrimination of transformants based on the implementation of conditions, other types of markers including screenable

markers such as GFP, whose basis is colorimetric analysis, are also contemplated. Alternatively, screenable enzymes may be utilized. One of skill in the art would also know how to employ immunologic markers, possibly in conjunction with FACS analysis. The marker used is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable and screenable markers are well known to one of skill in the art.

[0083] The invention may be better understood with reference to the following examples, which are representative of some of the embodiments of the invention, and are not intended to limit the invention.

EXAMPLE 1

[0084] **Optimized Plasmid Backbone.** One aspect of the current invention is the optimized plasmid backbone. The new synthetic plasmids presented below contain eukaryotic sequences that are synthetically optimized for species specific mammalian transcription. An existing pSP-HV-GHRH plasmid ("pAV0125") (SeqID#1), as shown in Figure 1 was synthetically optimized to form a new plasmid ("pAV0201")(SeqID#2). The plasmid pAV0125 was described in U.S. Patent 6,551,996 that was issued on April 23, 2003 titled "Super Active Porcine Growth Hormone Releasing Hormone Analog" with Schwartz, et al., listed as inventors, ("the Schwartz '996 Patent"). This 3,534 bp plasmid pAV0125 (SeqID #1) contains a plasmid backbone with various component from different commercially available plasmids, for example, a synthetic promoter SPc5-12 (SeqID #15), a modified porcine GHRH sequence (SeqID #4), and a 3'end of human growth hormone (SeqID #10). The new optimized synthetic expression vector (SeqID #2) contains 2,739 bp and is shown in Figure 2. The therapeutic encoded gene for the optimized plasmid in Figure 2 may also include optimized nucleic acid sequences that encode the following modified GHRH molecules.

ENCODED GHRH AMINO ACID SEQUENCE

wt-GHRH YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGERNQEQGA-OH

HV-GHRH HVDAIFTNSYRKVLAQLSARKLLQDILNRQQGERNQEQGA-OH

TI-GHRH YIDAIFTNSYRKVLAQLSARKLLQDILNRQQGERNQEQGA-OH

TV-GHRH YVDAIFTNSYRKVLAQLSARKLLQDILNRQQGERNQEQGA-OH

15/27/28-**GHRH** YADAIFTNSYRKVLAQLSARKLLQDILNRQQGERNQEQGA-OH

[0085] In general, the encoded GHRH or functional biological equivalent thereof is of formula:

$-A_1-A_2$–DAIFTNSYRKVL-A_3–QLSARKLLQDI-A_4-A_5–RQQGERNQEQGA-OH

wherein: A_1 is a D-or L-isomer of an amino acid selected from the group consisting of tyrosine ("Y"), or histidine ("H"); A_2 is a D-or L-isomer of an amino acid selected from the group consisting of alanine ("A"), valine ("V"), or isoleucine ("I"); A_3 is a D-or L-isomer of an amino acid selected from the group consisting of alanine ("A") or glycine ("G"); A_4 is a D-or L-isomer of an amino acid selected from the group consisting of methionein ("M"), or leucine ("L"); A_5 is a D-or L-isomer of an amino acid selected from the group consisting of serine ("S") or asparagines ("N").

[0086] An example of this new optimized synthetic expression vector was denoted as pAV0201 (SeqID#2). In order to construct pAV0201 (SeqID#2), the unwanted sequences from the pAV0125 (SeqID#1) were initially removed. A software program called Vector NTI (version 7.0) was used to generate and match sequences that could be compared and were known to be extraneous (e.g. LacZ promoter). There are many programs such as Vector NTI (version 7.0) that are known in the art and could have been used with similar results to compare and identify specific nucleic acid sequences. Once the extraneous DNA sequences were identified in the pAV0125 plasmid, they were removed by from the plasmid creating a truncated-pAV0125 plasmid. The Gene Forge® optimized synthetic sequences were used to produced codon frequencies that were matched in target and host organisms to ensure proper

folding. Gene Forge® was also used to identify and correct a number of deleterious structural elements in the relevant nucleic acid sequences. For example, a bias of GC content can be used to increase mRNA stability or reduce secondary structures; tandem repeat codons or base runs that may impair the gene can be minimized with codon optimization; modification of ribosome binding sites and mRNA degradation sites can be utilized; codon optimization can also reduce or eliminate problem secondary structures within the transcribed mRNA. Although Gene Forge® is a proprietary product of Aptagen that speeds codon optimization analysis, publicly available databases are available that allow a person with average skill in the art to replicate codon optimization protocol.

[0087] The pAV0125 plasmid contained a human Growth Hormone poly adenylation region that was approximately 618 bp. The original 618 bp region contained multiple poly adenylation sites and was reduced to only one. As a result over 400 bp were removed to an optimized length of 190bp (SeqID #10). Another 210bp poly A site is SeqID #16. The origin of replication (SeqID #12) was not altered.

[0088] A summary of the changes made to the pAV0125 plasmid backbone changes are as follows:

1. Although not wanting to be bound by theory, CpG islands are known to enhance immune responses, and are used to boost immune responses in vaccines (Manders and Thomas, 2000; McCluskie et al., 2000; Scheule, 2000)), the Gene Forge® system can identified and removed as many CpG island as possible without changing the translated amino acid sequence. Additionally, a Nco I site was removed from the Kanamycin sequence without altering the amino acid sequence. Currently the NcoI is an unique site, which makes the plasmid backbone more versatile.

2. The lacZ promoter region that was located downstream of the hGH polyA site was determined to be unnecessary, and it was subsequently removed.

3. A portion of the hGH polyA region was removed to produce a more compact plasmid that is able to accommodate longer DNA fragments or transgenes.

4. A 118bp portion of the lacZ coding sequence that was located between the KanR gene and the C5-12 synthetic promoter was determined to be unnecessary, and it was subsequently removed.

[0089] As a result of the above modifications to the plasmid backbone, a new synthetic plasmid as shown in Figure 2 was constructed. The pAV0201 optimized plasmid comprises a 2,739 bp circular plasmid (SeqID#2). The pAV0201 plasmid contains at least one eukaryotic coding region, and at least one prokaryotic coding sequence, wherein it has been contemplated that the eukaryotic coding region contains a modified growth hormone releasing hormone ("GHRH"). The pAV0201 plasmid also contains a poly A signal, wherein the human growth hormone poly A has been utilized. The pAV0201 plasmid also contains a eukaryotic promoter, and it has been contemplated that the c5-12 synthetic eukaryotic promoter of skeletal actin will be used, although other may be equally useful. The pAV0201 also contains a prokaryotic promoter. The prokaryotic promoter is PNEO, and a 19-47 bp sequence of transposon fragment ("Tn5") with accession number V00618. Additionally one NEO ribosome binding site ("RBS") is present in the pAV0201 plasmid. A complementary

origin of replication sequence ("pUC ori") from the pUC18 plasmid (e.g. 685-1466 bp of pUC18). A 5' untranslated region ("5' UTR") was inserted into the pAV0201 plasmid. The 5' UTR is from human growth hormone hGH 5' UTR (i.e. 504-557 bp) accession number M13438.

EXAMPLE 2

[0090] Optimized Synthetic GHRH sequences. Another aspect of the current invention is to utilize the above optimized plasmid backbone (pAV0201) and insert codon optimized species specific eukaryotic nucleic acid expression sequences. Although not wanting to limit the scope of the invention, five novel species of optimized GHRH nucleic acid sequences have been inserted into the pAV0201 plasmid backbone using the Nco I and Hind III restriction sites. Each sequence was codon optimized for expression in the corresponding species. The corresponding species in the below examples are as follows: mouse; rat; bovine; ovine; and chicken. The selection of these 5 species is not intended to limit the scope of species specific GHRH insertions into the pAV0201 plasmid backbone. In addition the structural features of pAV0201, each eukaryotic expression sequence also contains a signal peptide sequence for the purpose of making a signal peptide upstream from the mature peptide. Each signal peptide sequence has been contemplated to be the appropriate for the specific species of interest. However, in one example below (e.g. the chicken GHRH sequence), the rat GHRH signal peptide has been utilized. While the natural cDNA sequences (Baird et al., 1986) are known for mouse (Frohman et al., 1989b), rat (Bohlen et al., 1984; Mayo et al., 1985), ovine (Brazeau et al., 1984), bovine (Esch et al., 1983), porcine (Bohlen et al., 1983), chicken (McRory et al., 1997), the codon optimization expression sequences in conjunction with the pAV0201 based plasmid backbone make each of the constructs entirely unique.

[0091] One aspect of the current invention is the insertion of the codon optimized nucleic acid expression sequence for mouse GHRH ("mGHRH") (SeqID#5) into the pAV0201 plasmid backbone to give pAV0202 (SeqID#17). A schematic representation of the optimized nucleic acid expression sequence for mGHRH is shown in Figure 4. The optimized 228bp mGHRH fragment was sub-cloned into the pAV0201 vector using the Nco I and Hind III restriction enzyme cut sites, and standard methods known to one with ordinary skill in the

art of molecular biology. Figure 5 shows a detailed nucleic acid and amino acid sequence of the mGHRH motif, wherein all changes to the nucleic acid expression sequences are labeled in bold. The nucleic acid alignment between the original sequence (GHRH-M Ori) and Gene Forge optimized sequence (GHRH-M Opti) are shown in Figure 6, changes are labeled in bold. Figure 7 shows a comparison to indicate that the amino acid sequence has not changed due to codon optimization.

[0092] Another aspect of the current invention is the insertion of the codon optimized nucleic acid expression sequence for rat GHRH ("rGHRH") (SeqID#6) into the pAV0201 plasmid backbone to give pAV0203 (SeqID#18). A schematic representation of the optimized nucleic acid expression sequence for rGHRH is shown in Figure 8. The optimized 231bp rGHRH fragment was sub-cloned into the pAV0201 vector using the Nco I and Hind III restriction enzyme cut sites, and standard methods known to one with ordinary skill in the art of molecular biology. Figure 9 shows a detailed nucleic acid and amino acid sequence of the rGHRH motif, wherein all changes to the nucleic acid expression sequences are labeled in bold. The nucleic acid alignment between the original sequence (GHRH-R Ori) and Gene Forge optimized sequence (GHRH-R Opti) are shown in Figure 10, changes are labeled in bold. Figure 11 shows a comparison to indicate that the amino acid sequence has not changed due to codon optimization.

[0093] Another aspect of the current invention is the insertion of the codon optimized nucleic acid expression sequence for bovine GHRH ("bGHRH") (SeqID#7) into the pAV0201 plasmid backbone to give pAV0204 (SeqID#19). A schematic representation of the optimized nucleic acid expression sequence for bGHRH is shown in Figure 12. The optimized 222bp bGHRH fragment was sub-cloned into the pAV0201 vector using the Nco I and Hind III restriction enzyme cut sites, and standard methods known to one with ordinary skill in the art of molecular biology. Figure 13 shows a detailed nucleic acid and amino acid sequence of the bGHRH motif, wherein all changes to the nucleic acid expression sequences are labeled in bold. The nucleic acid alignment between the original sequence (GHRH-B Ori) and Gene Forge optimized sequence (GHRH-B Opti) are shown in Figure 14, changes are labeled in bold. Figure 15 shows a comparison to indicate that the amino acid sequence has not changed due to codon optimization.

[0094] Another aspect of the current invention is the insertion of the codon optimized nucleic acid expression sequence for ovine GHRH ("oGHRH") (SeqID#8) into the pAV0201 plasmid backbone to give pAV0205 (SeqID#20). A schematic representation of the optimized nucleic acid expression sequence for oGHRH is shown in Figure 16. The optimized 222bp oGHRH fragment was sub-cloned into the pAV0201 vector using the NcoI and Hind III restriction enzyme cut sites, and standard methods known to one with ordinary skill in the art of molecular biology. Figure 17 shows a detailed nucleic acid and amino acid sequence of the oGHRH motif, wherein all changes to the nucleic acid expression sequences are labeled in bold. The nucleic acid alignment between the original sequence (GHRH-O Ori) and Gene Forge optimized sequence (GHRH-O Opti) are shown in Figure 18, changes are labeled in bold. Figure 19 shows a comparison to indicate that the amino acid sequence has not changed due to codon optimization.

[0095] Another aspect of the current invention is the insertion of the codon optimized nucleic acid expression sequence for chicken GHRH ("cGHRH") (SeqID#9) into the pAV0201 plasmid backbone to give pAV0206 (SeqID#21). A schematic representation of the optimized nucleic acid expression sequence for cGHRH is shown in Figure 20. The optimized 234bp cGHRH fragment was sub-cloned into the pAV0201 vector using the Nco I and Hind III restriction enzyme cut sites, and standard methods known to one with ordinary skill in the art of molecular biology. Figure 21 shows a detailed nucleic acid and amino acid sequence of the cGHRH motif, wherein all changes to the nucleic acid expression sequences are labeled in bold. The nucleic acid alignment between the original sequence (GHRH-C Ori) and Gene Forge optimized sequence (GHRH-C Opti) are shown in Figure 22, changes are labeled in bold. Figure 23 shows a comparison to indicate that the amino acid sequence has not changed due to codon optimization. For this particular sequence, the chicken pre-pro hormone signal was replaced with the more compact, shorter rat pre-pro sequence.

[0096] The above optimized plasmid constructs can be administered to a mammalian host for various therapeutic effects. One skilled in the art recognizes that different methods of delivery may be utilized to administer an optimized synthetic expression vector into a cell. Examples include: (1) methods utilizing physical means, such as electroporation (electricity), a gene gun (physical force) or applying large volumes of a liquid

(pressure); and (2) methods wherein said vector is complexed to another entity, such as a liposome or transporter molecule.

[0097] Accordingly, the present invention provides a method of transferring a therapeutic gene to a host, which comprises administering the vector of the present invention, preferably as part of a composition, using any of the aforementioned routes of administration or alternative routes known to those skilled in the art and appropriate for a particular application. Effective gene transfer of a vector to a host cell in accordance with the present invention to a host cell can be monitored in terms of a therapeutic effect (*e.g.* alleviation of some symptom associated with the particular disease being treated) or, further, by evidence of the transferred gene or expression of the gene within the host (*e.g.*, using the polymerase chain reaction in conjunction with sequencing, Northern or Southern hybridizations, or transcription assays to detect the nucleic acid in host cells, or using immunoblot analysis, antibody-mediated detection, mRNA or protein half-life studies, or particularized assays to detect protein or polypeptide encoded by the transferred nucleic acid, or impacted in level or function due to such transfer).

[0098] These compositions and methods described herein are by no means all-inclusive, and further methods to suit the specific application will be apparent to the ordinary skilled artisan. Moreover, the effective amount of the compositions can be further approximated through analogy to compounds known to exert the desired effect.

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The entire content of each of the following U.S. patent, foreign patent and publication documents is incorporated by reference herein.

U.S. PATENT DOCUMENTS

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U.S. Patent No. 5,134,120 issued on July 28, 1992 and titled "Use of growth hormone to enhance porcine weight gain" with Boyd, et al., listed as inventors.

U.S. Patent No. 5,061,690 issued on October 29, 1991 and titled "Method for increasing milk production in mammals and/or increasing the birth weight of their newborn and improving postnatal growth " with Kann, et al., listed as inventors.

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